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Effect of apoferritin surface-biomacromolecular modification on cellular uptake and inhibition of protein corona

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Abstract: The effects of surface modifications (PEGylation and PASylation) of natural nanocarriers based on apoferritin (FRT) were tested in this work. The main goals of performed PEGylation/PASylation were decreased protein corona formation leading to better internalization of drugs into diseased cells and therefore higher efficiency of treatment. The influence of created protein coronas on the amount of internalized experimental drug ellipticine (Elli) was evaluated *via* fluorescence microscopy. Various properties of these modified nanoparticles were studied, such as their cytotoxicity or release kinetics of Elli. According to performed experiments, PAS-10 modification appeared as the most appropriate surface modification.

Key Words: apoferritin, nanomedicine; PASylation, PEGylation, protein coronas

INTRODUCTION

One of the most limiting factors of a nanocarrier use in therapy is immediate binding of plasma proteins on their surface upon entering blood stream. This process is known as protein corona formation. Protein corona can be defined as a natural interface between nanomaterials and living matter in biological milieu (Monopoli et al. 2013). It is known that corona formation could affect endocytosis or functional properties of nanocarrier (Yan et al. 2013). The most important fact is that, according to Salvati et al., corona interferes with targeting moieties, which leads to inhibited receptor-mediated uptake of the nanocarriers (Salvati et al. 2013). Moreover, protein coronas, which were taken up by target cells, could alter cells functions (Bros et al. 2018).

To minimalize or completely inhibit binding of additional biomolecules leading to formation of protein corona it is possible to modify the surface of nanocarrier. In this contribution, we focused on two very promising surface modifications: PEGylation and PASylation. PEGylation is generally defined as modification of proteins, peptides or small organic molecules by covalent binding with one or more poly-ethylene glycol (PEG) chains (Eto et al. 2008). PEG is approved by Food and Drug Administration (FDA) for human oral, intravenous and dermal pharmaceutical use (Li et al. 2013). Despite this fact, the use of PEGylation has some drawbacks including potential immunogenic effects (Armstrong et al. 2007), non-biodegradability of PEG, possible cellular vacuolization or decreased biological activity of a drug after performed PEGylation (Yu et al. 2007). Therefore, we also focused on PASylation, which represents biological alternative to PEGylation. PAS sequences are hydrophilic, uncharged, comprising of small residues of amino acids proline, alanine and (Binder et al. 2017).

As a nanocarrier we chose FRT, which is a biocompatible ubiquitous protein naturally occurring in human body (Bulvik et al. 2012). The structure of FRT is pH dependent. In our Research group we took advantage of this fact and published a study, where we described easy-to-use encapsulation of cytostatic drug doxorubicin (Dox) into FRT (Dostalova et al. 2017). For the purpose of this study was

used experimental cytotoxic drug Elli. Elli has not been approved by FDA due to many side effects, such as its mutagenicity or hemotoxicity (Stiborova et al. 2011). Elli is classified as an alkaloid, which mechanism of action is presumably based on intercalation into DNA and inhibition of topoisomerase II (Tmejova et al. 2014).

By encapsulation of Elli into FRT we show that even experimental drugs can be used as cargoes for FRT-based delivery with pronounced cytotoxic effects. By modifying the surface of FRT with PEGylation or PASylation, we increased the internalization of Elli into cells and also decreased the formation of protein corona. Furthermore, we also evaluated cytotoxic effects *via* MTT assay and we also studied one of the possible mechanism of toxicity, which is formation of reactive oxygen species (ROS).

MATERIAL AND METHODS

Chemicals

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Encapsulation of Elli into FRT

The stock solution of Elli with concentration of 1 mg/mL was prepared by dissolving Elli in 1 M HCl and deionized water in ratio 1 : 150. For each sample, 200 µL of 1 mg/mL Elli was added to 100 µL of deionized water and 20 µL of 50 mg/mL horse spleen FRT and gently mixed for 15 min. To reassemble the FRT structure disassembled by acidic Elli and encapsulate Elli in FRT cavity, 0.66 µL of 1 M sodium hydroxide solution was added and the samples were mixed for further 15 min. To filter out non-encapsulated Elli, solution exchange was performed 3× (centrifugation at 6,000 *g* for 15 min). The concentration of encapsulated Elli was evaluated by absorbance measurement at 420 nm using Tecan Infinite 200 PRO (Männedorf, Switzerland).

Surface modification with PEG

50 µL of 10 mM PEG maleimide in PBS (phosphate buffered saline, pH 7.4: 0.137 M NaCl + 0.0027 M KCl + 0.0014 M KH_2PO_4 + 0.0043 M Na_2HPO_4) and 629 µL of PBS was added to FRTElli and mixed for 1 h. To remove unbound PEG, the sample was 5× diafiltrated using Amicon® Ultra 0-5 mL 50K Merck Millipore (Billerica, MA, USA) at 6,000 *g* for 15 min.

Surface modification with PAS

25 µL of 1.3 nm gold nanoparticles was added to FRTElli and the samples were mixed for 14 h to allow adsorption of Au nanoparticles to the charged amino acid residues on the surface of FRTElli nanoparticles (creating FRTElli-Au). Solution exchange was performed 2× to remove unbound Au nanoparticles. 3 µL of 1.25 mg/mL PAS-10 (ASPAAPAPASC) and PAS-20 (ASPAAPAPASPAAPAPSAPAC) was added to FRTElli-Au and the samples were incubated for 1 h at 45 °C to allow binding of cysteine to gold. Then, solution exchange was performed to remove unbound molecules of PAS peptides.

Short-term cytotoxicity of PEGylated/PASylated FRTElli

The cell viability of breast cancer cell lines MDA-MB-231, MDA-MB-468, MCF-7, T-47D, ZR-75-1 and nonmalignant cell line HBL-100 was assayed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to determine 24IC_{50} values. The suspension of 5,000 cells in medium (50 µL) was added to each well of 96-well plates, followed by incubation for 24 h at 37 °C with 5%CO_2 to ensure cell growth. After 24 h treatment, MTT dye solution (10 µL, 5 mg/mL in PBS) was added to each well and the mixture was incubated for further 3 h at 37 °C. After that, medium containing MTT solution was replaced with 99.9% dimethyl sulfoxide (100 µL to each well), incubated for 5 min and the absorbance of the samples was determined using Tecan Infinite 200 PRO (λ=570 nm). The experiments were performed in three independent repetitions.

Release kinetics study

Release kinetics study was performed in order to test the stability of PEGylated and PASylated FRTElli. Prepared nanoparticles were incubated in Ringer's solution (0.65% NaCl, 0.042% KCl, 0.025% $CaCl_2$, 0.02% sodium bicarbonate) at 37 °C. The mixture was centrifuged at 6000 g and 4 °C

for 15 min at various time points (0, 1, 2, 4, 8 and 24 h). The pellets containing the nanocarrier were resuspended in Ringer's solution. The total amount of released Elli was measured by absorbance measurement at 420 nm using Tecan Infinite 200 PRO.

ROS formation

For ROS formation assay, a suspension of 10,000 MCF-7 or HBL-100 cells in medium was added to each well of a 24-well plate. After overnight incubation, the cells were treated with FRTElli with modified surface ($24IC_{50}$, 6 h). After treatment, the cells were rinsed with PBS and directly used for analysis of ROS using CellROX® Green Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Hoechst 33342 was employed for nuclei counterstaining. Cells were visualized using the EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific).

Fluorescently labeled protein coronas

To label FRT, 4 µL of fluorescent dye cyanine 3 (Lumiprobe, Hannover, Germany) and 100 µL of nanoparticle's solution were added to 896 µL of 0.05 M borate buffer (pH = 8.5) and mixed for 1 h. To label fetal bovine serum (FBS), FBS was centrifuged at 22,000 *g* and 4 °C for 30 min to remove aggregates, followed by incubation of 500 µL of FBS with 8 µL of fluorescent dye cyanine 5 (Lumiprobe) and 492 µL of 0.05 M borate buffer (pH = 8.5).To remove unbound cyanine dye molecules, the samples were 5× diafiltrated using Amicon® Ultra 0-5 mL 100K Merck Millipore at 6,000 *g* for 15 min. After diafiltration the samples were adjusted to 100 µL and 400 µL of 10× diluted labeled FBS was added to them. The samples containing a mixture of fluorescently labeled FBS and PEGylated/PASylated FRTElli nanoparticles were incubated at 600 rpm and 37 °C for 35 min. To remove unbound proteins from nanoparticles, the samples were 5× centrifuged at 6,000 *g* for 15 min. Then, a suspension of 10,000 MDA-MB-468 cells in medium were added wells of a 24-well plate. After overnight incubation, the cells were treated with FRTElli with modified surface ($24IC_{50}$, 6 h). After treatment, the cells were rinsed with PBS and directly used for fluorescence microscopy. Hoechst 33342 was employed for nuclei counterstaining. Cells were visualized using the EVOS FL Auto Cell Imaging System obtained from Thermo Fisher Scientific.

RESULTS AND DISCUSSION

MTT assay (Figure 1) was performed in order to determine value of $24IC_{50}$. The value of $24IC_{50}$ is crucial for cytotoxicity evaluation of performed surface modifications. $24IC_{50}$ was determined for five malignant breast cell lines (MDA-MB-468, MDA-MB-231, MCF-7, T-47D, ZR-75-1) and for one non-malignant breast cell line (HBL-100). The results from MTT assay showed that surface modification with PEG was more cytotoxic than PASylation for all cell lines and also that PAS-10 is more cytotoxic than PAS-20, except for the cell line T-47D and ZR-75-1.

Figure 1 MTT assay performed on FRT with modified or unmodified surface

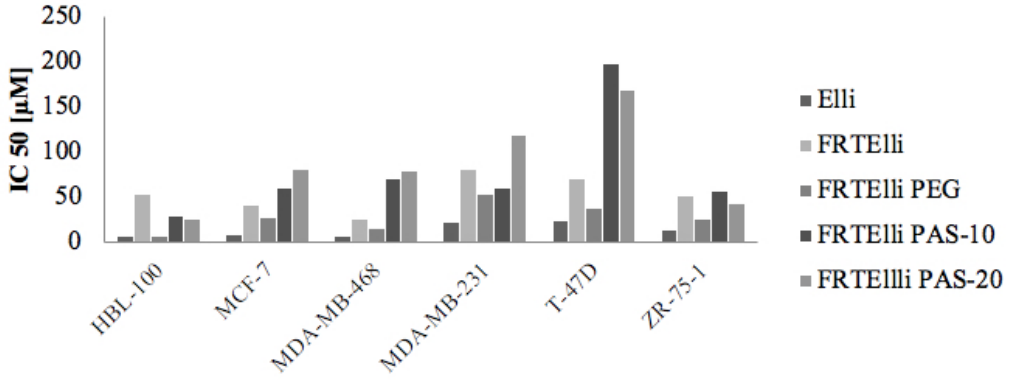
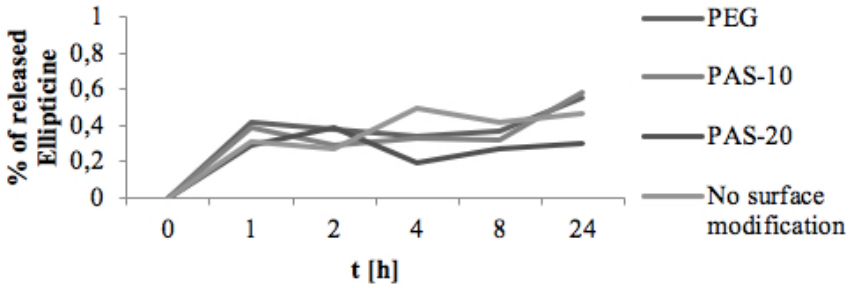


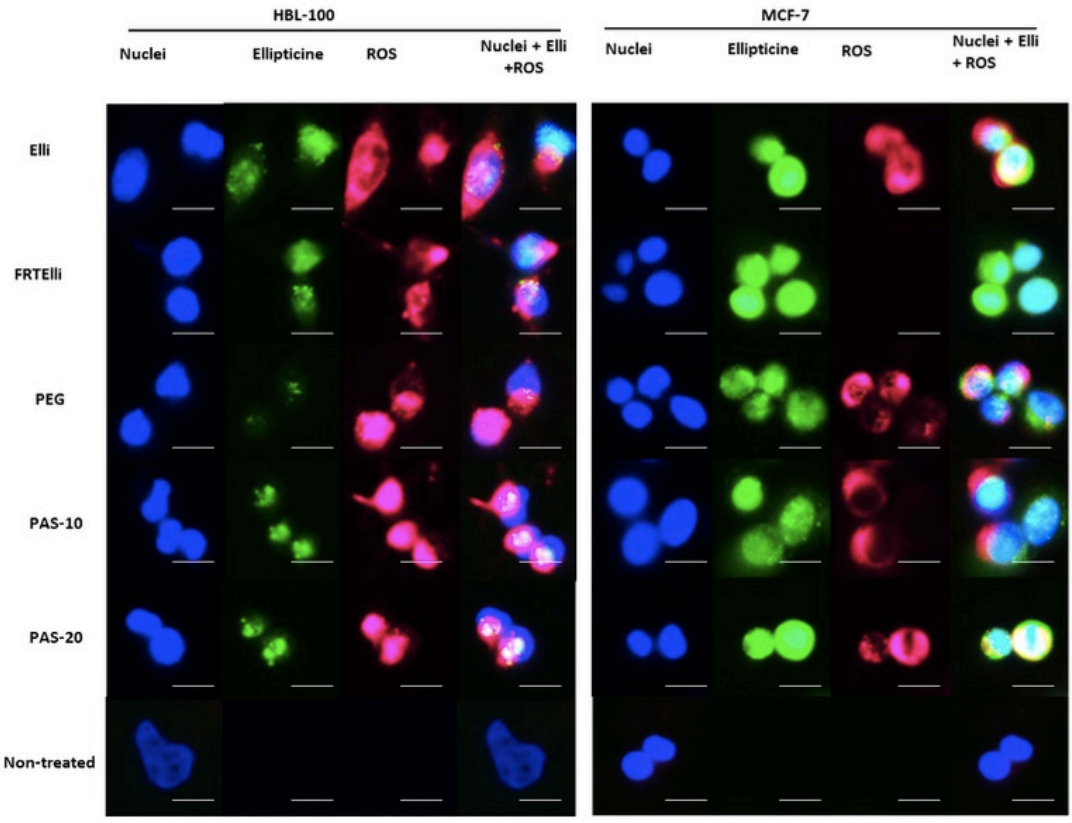
Figure 2 showed that neither PEGylation, nor PASylation caused any changes in the release kinetics in off-target plasma environment of Elli after 24 h study, showing exceptional stability of encapsulated Elli in plasma.

Figure 2 Release kinetics study on FRT with modified or unmodified surface.



The internalization of Elli into FRT is showed in Figure 3 with ROS/nuclei co-staining and fluorescence microscopy analysis of tested cell lines. Green color represents fluorescence of Elli. Figure 3 shows successful internalization of Elli into nuclei, especially to malignant cell line MCF-7. The internalization of Elli into non-malignant HBL-100 cells was 84% lower than into malignant cell line MCF-7. It must be noted that only surface modification with PAS-10 increased the internalization of Elli (166±6% compared to 100±4% of Elli), in case of PEGylation the internalization was 52% lower than for Elli and in case of PAS-20 5% lower than for Elli. Figure 3 illustrates that all three performed surface modifications induced increased formation of ROS compared to unmodified FRTElli within both tested cell lines, but, overall, the highest ROS formation was found in PAS-10 modified FRTElli for non-malignant cell line HBL-100 (218±8% compared to 100±4% of Elli) and in PAS-20 modified FRTElli for malignant cell line MCF-7 (269±9% compared to 100±4% of Elli). The fact that internalization of Elli is decreased for non-malignant cell line HBL-100, while the ROS formation is increased compared to malignant cell line MCF-7 suggest, that HBL-100 cells are more sensitive to Elli.

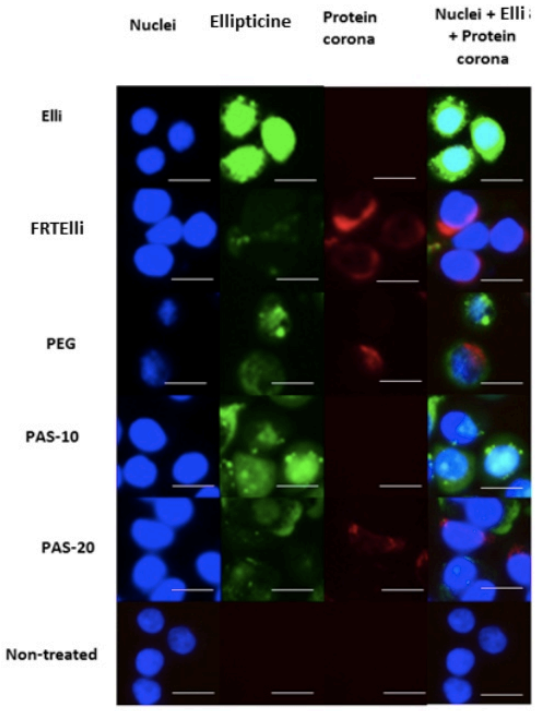
Figure 3 Internalization of Elli into FRT, ROS/nuclei staining (the length of the scale bar is 25 μm)



Before protein corona formation we fluorescently labeled FBS in order to further investigate the formation of protein coronas and then we performed fluorescence microscopy analysis of tested samples on MDA-MB-468 cell line. As it can be seen from Figure 4, red color represents proteins forming protein coronas, green color represents fluorescence of Elli and blue color represents stained nuclei. Figure 4

shows, that Elli internalized successfully also into nuclei of MDA-MB-468. It must be noted, that all three surface modification increased internalization of Elli compared to unmodified FRTElli. The highest internalization of Elli was noted on PAS-10 modified FRTElli, which was even higher than for non-encapsulated Elli (121±5% compared to 100±4% of Elli). PEGylated FRTElli had decreased internalization of Elli compared to PAS-10 or non-encapsulated Elli (82±3% compared to 100±4% of Elli). The lowest internalization was noticed for FRTElli with PAS-20 surface modification (48±2% compared to 100±4% of Elli). We also focused on evaluation of protein corona formation. As it can be seen, the highest protein corona formation was detected on FRTElli without any surface modification, which also corresponds to a fact, that there was observed the lowest degree of Elli internalization. On the other hand, almost no protein corona formation was observed on non-encapsulated Elli and FRTElli modified with PAS-10 (23±1% compared to 100±4% of FRTElli). PEGylation also caused significant decrease of protein corona (28±2% compared to 100±4% of FRTElli). Surface modification with PAS-20 appeared as the least useful due to insignificant decrease of protein corona (87±4% compared to 100±4% of FRTElli) and insufficient internalization of Elli.

Figure 4 Fluorescently labeled protein coronas on modified/unmodified surface of FRT (the length of scale bar is 25 μm)



CONCLUSION

The experiment presented in this work served as suitable platform for the prediction of *in vivo* behavior of FRT nanocarrier, based on *in vitro* tests of protein corona formation. The surface of FRT was modified with polymer (PEG) and peptides (PAS) in order to decrease negative interactions with surrounding environment. Overall, the results showed that decreased protein corona formation led to increased internalization of Elli. All three tested modifications favorably influenced the internalization of Elli and also protein corona formation, while as the most beneficial appeared the modification with PAS-10. To further determine the identity of proteins composing protein coronas, 2-D fluorescence difference gel electrophoresis (DIGE) followed by mass spectrometry (MS) analysis will be performed.

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